Protocol

Time-Lapse Imaging of Live *Phallusia* Embryos for Creating 3D Digital Replicas

François B. Robin, Delphine Dauga, Olivier Tassy, Daniel Sobral, Fabrice Daian, and Patrick Lemaire

During embryonic development, cell behaviors that are tightly coordinated both spatially and temporally integrate at the tissue level and drive embryonic morphogenesis. Over the past 20 years, advances in imaging techniques, in particular, the development of confocal imaging, have opened a new world in biology, not only giving us access to a wealth of information, but also creating new challenges. It is sometimes difficult to make the best use of the recordings of the complex, inherently three-dimensional (3D) processes we now can observe. In particular, these data are often not directly suitable for even simple but conceptually fundamental quantifications. This article provides a method to fluorescently label and image structures of interest that will subsequently be reconstructed, such as cell membranes or nuclei. The protocol describes live imaging of *Phallusia mammillata* embryos, which are robust, colorless, and optically transparent with negligible autofluorescence. Their diameter ranges from 100 µm to 120 µm, which allows time-lapse microscopy of whole embryos using two-photon microscopy with a high-resolution objective. Although two-photon imaging is described in detail, any imaging technology that results in a *z*-stack may be used. The resulting image stacks can subsequently be digitalized and segmented to produce 3D embryo replicas that can be interfaced to a model organism database and used to quantify cell shapes.

RELATED INFORMATION

Protocols are also available for **Imaging of Fixed *Ciona* Embryos for Creating 3D Digital Replicas** (Robin et al. 2011a) and **Creating 3D Digital Replicas of Ascidian Embryos from Stacks of Confocal Images** (Robin et al. 2011b).

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Artificial seawater-HEPES (ASWH) <R>

FM4-64 (Molecular Probes T3166)

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Tris (1 M, pH 9.5)

Trypsin (0.1%; Sigma-Aldrich)

Equipment

Glass-bottom dish, 35-mm-diameter plastic (WillCo Wells GWSt-3522)

Treat the dish with gelatin/formaldehyde (each 0.1%) and rinse thoroughly to prevent embryos from sticking. Silicon chambers may be used as an alternative (see Step 6).

Imaging system

- Two-photon inverted microscope (Zeiss LSM 510 NLO)
- 63× objective (C-Apochromat 1.2 W Corr; Carl Zeiss, Inc.) illuminated with a Mai-Tai laser (Spectra-Physics) at a wavelength of 1020 nm in a nondescanned detection (NDD) mode
- Cooling stage: Open perfusion microincubator (PDMI-2; Harvard Apparatus) with a temperature controller (TC-202A; Harvard Apparatus)
- Hardware and software as described in **Creating 3D Digital Replicas of Ascidian Embryos from Stacks of Confocal Images** (Robin et al. 2011b)

Petri dish (coated with 1% agarose)

Pipette (glass with 200-µm exit diameter)

Soak the pipette in water overnight to prevent embryos from sticking to the glass during manipulations.

METHOD

1. Set the imaging room temperature to ~19°C.

   The imaging chamber temperature needs to be adjusted because Phallusia *develops well between 16°C and 22°C.*
2. Dissect gravid *Phallusia* and collect eggs and sperm separately.
3. Dechorionate the embryos by incubating in an agarose-coated Petri dish containing 6 mL of 0.1% trypsin for ~2 h. Rinse four times in ASWH.
4. Activate the sperm by adding 1–5 µL of concentrated sperm to 1 mL of ASWH containing 50 µL of 1 M Tris (pH 9.5).
5. Fertilize the embryos by adding 50 µL of activated sperm in 3 mL of ASWH. After 10 min, transfer the embryos to a new dish to prevent polyspermy.

   For detailed protocol, refer to McDougall and Sardet (1995).
6. About 30 min before imaging, use a pipette with a 200-µm exit diameter to transfer the developing embryos to a glass-bottom dish (pretreated with 0.1% gelatin/formaldehyde) and containing 5 µg of FM4-64 in 1 mL of ASWH.

   Constant presence of the dye ensures the membranes remain labeled, thereby reducing the impact of photobleaching and membrane recycling. Alternatively, use silicon chambers to image embryos under different conditions simultaneously (e.g., in the presence of a drug and control). Prevent evaporation by covering the sample with a cover glass.
7. Move the sample to the inverted two-photon microscope. Select embryos with an equatorial plane parallel to the surface of the dish (and hence to the imaging plane).
8. Adjust parameters to optimize a trade-off between image quality (signal/noise ratio), spatial resolution, and imaging time.

   i. Spatial resolution: Test the full reconstruction process with sample data to ensure it matches an adequate spatial resolution for the object to be reconstructed (go to http://aniseed-ibdm.univ-mrs.fr/ for example stacks and more information). The quality of the reconstruction is highly dependent on spatial resolution. We routinely use a voxel size (x–y–z) of $0.394591 \times 0.39 \times 3$ µm^3 with a $512 \times 512 \times 50$ sample size or, when higher resolution is needed along the z-axis, $0.39 \times 0.39 \times 1$ µm^3 voxel size with a $512 \times 512 \times 150$ sample size.

ii. Exposure time: Two conflicting requirements need to be balanced. First, early ascidian embryos develop very rapidly with a cell-cycle time of <30 min. In addition, cell shapes change dramatically within a single cell cycle (Tassy et al. 2006). Time to image an embryo from top to bottom should therefore not exceed 3 min at 19°C. Second, use of a high laser power leads to photobleaching and phototoxicity. To image in an NDD mode, we routinely set the laser power at ~30% of the 500 mW output power at 1000 nm. With such settings and a voxel size of $0.39 \times 0.39 \times 3$ μm^3, the imaging of a single stack lasts for ~150 sec.

iii. Frequency of imaging: When recording a time-lapse movie of embryo development, we usually generate a stack every 7 min. Using this frequency, we can image single embryos through cleavage and gastrulation (2 h of recordings corresponds to 20 stacks) (see online Movie 1 at http://cshprotocols.cshlp.org/).

If your setup is equipped with a motorized stage, it is possible to image several embryos simultaneously, sequentially switching from one embryo to the next at each developmental stage. Care should be taken to slow down the speed of the motorized stage and fix the dish tightly to the stage to ensure there is no drift during imaging. As the objective visits the imaged positions, the oil spreads on the slide. Add oil on every location you image to make sure the oil film does not get too thin when the objective moves.

DISCUSSION

In contrast to *Phallusia*, embryos of the broadly studied species *Ciona intestinalis* are pink, rather opaque, and show substantial autofluorescence, particularly in the muscle lineages. As a result, two-photon imaging can only be performed up to 30 µm deep in live *C. intestinalis* embryos. In this species, therefore, whole embryo imaging is performed on fixed and optically cleared specimens stained with Hoechst and phalloidin on a regular or two-photon confocal microscope as described in **Imaging of Fixed *Ciona* Embryos for Creating 3D Digital Replicas** (Robin et al. 2011a).

RECIPE

Artificial seawater-HEPES (ASWH)

Reagent	Amount to add for 1 L	Final concentration
NaCl	24.55 g	420 mM
KCl	0.67 g	9 mM
$CaCl_2 \cdot 2H_2O$	1.47 g	10 mM
$MgCl_2 \cdot 6H_2O$	4.48 g	24.5 mM
$MgSO_4 \cdot 7H_2O$	6.29 g	25.5 mM
$NaHCO_3$	0.18 g	2.15 mM
HEPES (1 M, pH 8.0)	5 mL	5 mM

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